Isolation and Characterization of Two Novel Antibacterial Cyclic Hexapeptides from *Streptomyces alboflavus* 313

by **Zhiqin Ji***[a]), **Gang Qiao**[a]), **Shaopeng Wei**[b]), **Lixia Fan**[b]), and **Wenjun Wu**[b])[c])

[a]) State Key Laboratory of Crop Stress Biology in Arid Areas, Northwest A&F University, Yangling, Shaanxi 712100, P. R. China (phone: +86-29-87093987; fax: +86-29-87093987; e-mail: jizhiqin@nwsuaf.edu.cn)

[b]) Shaanxi Province Key Laboratory of Research and Development on Botanical Pesticide, Northwest A & F University, Yangling, Shaanxi 712100, P. R. China

[c]) Key Laboratory of Plant Protection Resources and Pest Integrated Management, Ministry of Education, College of Plant Protection, Northwest A & F University, Yangling, Shaanxi 712100, P. R. China

Two novel cyclic hexapeptides, designated NW-G08 (**1**) and NW-G09 (**2**), were isolated from the fermentation broth of *Streptomyces alboflavus* 313. Their structures were elucidated on the basis of extensive spectroscopic analysis, MS experiments, and chemical analysis. Their antibacterial activities against several strains of bacteria were evaluated by micro-broth dilution method. NW-G08 (**1**) and NW-G09 (**2**) were highly antibacterial against *Gram*-positive but not active against *Gram*-negative bactaria. The minimum inhibitory concentrations (*MIC*s) of **1** against *Bacillus cereus*, *Bacillus subtilis*, and *Staphylococcus aureus* were 6.25, 6.25, and 1.56 μg/ml, respectively. It is worth noting that **1** and **2** exhibited much higher activities against methicillin-resistant *Staphylococcus aureus* (MRSA) than ampicillin, which implies that they might be potential candidates for the development of novel drugs against resistant pathogenic bacteria.

Introduction. – Peptides are the largest chemical group of natural antibiotics produced by microorganisms, in which cyclic peptides have attracted more attention due to the diversity of their biological activities [1]. Since monamycins were first isolated from *Streptomyces jamaicensis* in 1995 [2], a series of natural compounds which contain the piperazine-3-carboxylic acid moiety have been found among the metabolites of microorganisms, such as azinothricins [3–6], chloptosin [7], himastatin [8–10], and piperazimycin [11]. These natural products exhibit impressive antimicrobial, antitumor and anti-HIV activities. Attracted by their intriguing chemical structure and a medicinally relevant biological activity profile, the piperazic acid-containing natural products mentioned above have been successfully synthesized in recent years [12].

In the previous investigation, our group also isolated a piperazic acid-containing cyclic hexapeptide, NW-G01, from the fermentation broth of *Streptomyces alboflavus* 313 [13–15]. NW-G01 exhibited strong antimicrobial activity against *Gram*-positive bacteria. The structures of himastatin, chloptosin, and NW-G01 consist of valine (Val), piperazic acid (PA), and a pyrroloindoline derivative (Trp). Although they are significantly different in the amino acid content, their antimicrobial activities are similar. All the compounds could selectively inhibit *Gram*-positive bacteria, including

methicillin-resistant *Staphylococcus aureus* (MRSA), whereas they are not active against *Gram*-negative bacteria. In our further investigation, two novel cyclic hexapeptides, NW-G08 (**1**) and NW-G09 (**2**), were isolated from the producer of NW-G01 by large-scale fermentation (see *Fig. 1*). We report here the structure elucidation and antimicrobial activity of these cyclic hexapeptides.

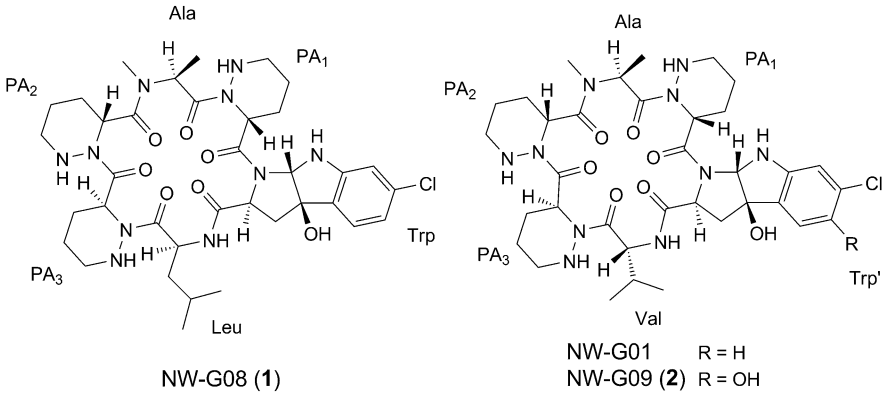


Fig. 1. *Structures of NW-G01, NW-G08* (**1**)*, and NW-G09* (**2**)

Results and Discussion. – *Chemistry. NW-G08* (**1**). The IR spectrum of **1** exhibited typical absorption bands of amide CO groups at 1660 cm^{-1}. Its molecular formula was established as $C_{36}H_{51}ClN_{10}O_7$ by HR-ESI-MS (*m/z* 771.3716, ($[M+H]^+$, $C_{36}H_{52}ClN_{10}O_7^+$; calc. 771.3709). The presence of the Cl-atom was suggested by the isotope abundance peaks in the MS spectrum. The ^{13}C-NMR and DEPT spectra (*Table 1*) of **1** showed 36 C-atom signals, which were attributed to four Me, eleven CH_2, and eight sp^3CH groups, one O-bearing sp^3 quaternary C-atom, three sp^2-CH groups, three sp^2 quaternary C-atoms, and six amide CO C-atoms. ^{1}H- and ^{13}C- chemical-shift assignments were accomplished by standard 1D- and 2D-NMR techniques, such as DEPT, HSQC, and HMBC. The ^{1}H-NMR spectrum of **1** exhibited signals of two Me groups at δ(H) 3.00 (*s*) and 1.34 (*d*, $J=6.5$), and of one CH group at δ(H) 5.75 (*d*, $J=$ 6.5), which were assigned to the MeN H-atoms, and H_β- and H_α-atoms of *N*-methylalanine, respectively, based on the HSQC and HMBC spectra of **1**, and by comparison with the corresponding chemical shifts and coupling constants of NW-G01 [14]. The characteristic signals of CH groupat δ(H/C) 5.49/85.6, of O-bearing quaternary C-atom at δ(C) 88.4, of three aromatic CH groups at δ(H/C) 6.73/110.7, 6.81/119.2, 7.22/123.7, and three sp^2 quaternary C-atoms at δ(C) 129.5, 135.0, and 149.2 indicated the presence of 6-chloro-1,2,3,3a,8,8a-hexahydro-3a-hydroxy-pyrrolo-[2,3-*b*]indole-2-carboxylic acid; the chlorinated pyrroloindoline moiety usually was considered to be derived from tryptophan (Trp). Three molecules of piperazic acid were also identified based on the HSQC and HMBC spectra of **1**, and by comparison with the corresponding chemical-shift and coupling-constant values of NW-G01. The signals of two CH groups at δ(H) 5.13–5.15 (*m*) and 1.55–1.58 (*m*), of two Me groups at δ(H) 0.96 (*d*, $J=7.0$) and 0.98 (*d*, $J=7.0$), and of one CH_2 group at δ(H) 1.59–1.61

and 1.40–1.42 (*m*) could be assigned to the H_α, H_γ, H_δ, $H_{\delta'}$, and H_β of leucine based on ^{1}H,^{1}H-COSY and HMBC correlations (see *Table 1*). Thus, the structure of **1** was established as consisting of *N*-methylalanine (MeAla), 6-chloro-1,2,3,3a,8,8a-hexahydro-3a-hydroxy-pyrrolo[2,3-*b*]indole-2-carboxylic acid (Trp), leucine (Leu), and three molecules of piperazic acid (PA).

The sequence linkage of the amino acids in **1** could be readily determined by comparison of the characteristic MS/MS fragmention peaks in the spectra of **1** and NW-G01 (*Table 2*, *Scheme 1*, and *Fig. 2*). Fragmentation pathway of NW-G01 was illustrated first, the product ions were derived mostly from the cleavage of peptide bonds, and the amino acid content of important fragments were also deduced. Based on the fragmentation pathway, fragmention peaks at *m*/*z* 672, 658, 533, 448, 409, 349, and 310 observed in the MS/MS spectrum of NW-G01 could be attributed to PA_1-Trp-Val-PA_3-PA_2, PA_3-PA_2-Ala-PA_1-Trp, MeAla-PA_1-Trp-Val, PA_1-Trp-Val, Val-PA_3-PA_2-Ala, PA_1-Trp, and PA_2-MeAla-PA_1, respectively. At the same time, fragmention peaks at *m*/*z* 658, 349, and 310 also appeared in the MS/MS spectrum of **1**, indicating that the sequence linkage of the amino acids in **1** was identical with that in NW-G01, except valine was replaced with leucine. This could be confirmed by further characteristic fragmention peaks appearing in the spectrum of **1**. Fragmention peaks at *m*/*z* 686, 547, 462, and 423 were observed in the spectrum of **1**, indicating that each fragment was 14 Da more than the corresponding fragments of PA_1-Trp-Val-PA_3-PA_2, MeAla-PA_1-Trp-Val, PA_1-Trp-Val, Val-PA_3-PA_2-Ala revealed in the spectrum of NW-G01, the molecular weight of leucine is 14 Da higher than that of valine, so these ions could be elucidated as PA_1-Trp-Leu-PA_3-PA_2, MeAla-PA_1-Trp-Leu, PA_1-Trp-Leu, and Leu-PA_3-PA_2-Ala. In other words, the *m*/*z* values of Leu-containing fragments observed in the spectrum of **1** were 14 Da higher than those Val-containing fragments observed in that of NW-G01, and the *m*/*z* value of fragment in which Leu was not presented was equal to that of NW-G01. The conclusion could also be supported by the results of the HMBC experiment, which showed correlation from the α-CH group H-atoms, particular of amino acid residues, to the CO C-atom of the neighboring residues.

The absolute configurations of the amino acids were determined by application of the *Marfey*'s method as we described previously [14], as well as the NOESY spectrum. By comparing the retention times of their 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA) derivatives with the purchased L-leucine and that of NW-G01, the absolute stereochemistry of amino acid in **1** was determined as L-leucine, *N*-methyl-D-alanine, (*3R*)-piperazic acid (PA_1), (*3R*)-piperazic acid (PA_2), (*3S*)-perazic acid (PA_3), and(2*S*,3a*R*,8a*S*)-6-chloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-[2,3-*b*]indole-2-carboxylic acid (*Fig. 1*).

NW-G09 (**2**). The IR spectrum of **2** exhibited typical absorption bands of amide CO groups at 1636 cm^{-1}. Its molecular formula was established as $C_{35}H_{49}ClN_{10}O_8$ by HR-ESI-MS (*m*/*z* 773.3511 ($[M+H]^+$, $C_{35}H_{50}ClN_{10}O_8^+$; calc. 773.3502). The presence of the Cl-atom was evidenced by the isotope abundance peaks in the MS spectrum. The ^{13}C-NMR and DEPT spectra (*Table 3*) of **2** showed 35 C-atom signals, which were recognized as those of four Me, ten CH_2, and eight sp^3 CH groups, one O-bearing sp^3 quaternary C-atom, two sp^2 CH groups, and four sp^2 quaternary and six amide CO C-atoms. ^{1}H- and ^{13}C-chemical-shift assignments were achieved by standard 1D- and 2D-NMR techniques, such as DEPT, HSQC, and HMBC. The ^{1}H-NMR spectrum of **2**

Table 1. *The ^{1}H- and ^{13}C-NMR Chemical Shifts of NW-G08*[a]) (**1**; CD_3OD, ^{1}H: 500 MHz, ^{13}C: 125 MHz). δ in ppm, J in Hz.

Position	δ(C)	δ(H)	^{1}H,^{1}H-COSY	HMBC	NOESY
Leu					
CO	174.7			α, PA_3-α	
α	48.9	5.13–5.15 (*m*)	β	β, γ	β
β	42.0	1.59–1.61 (*m*, H_a)	α, β_2, γ		
		1.40–1.42 (*m*, H_b)	α, β_1, γ	α, γ	
γ	24.9	1.55–1.58 (*m*)	β, γ	β, δ	
δ	19.7	0.98 (*d*, *J* = 7.0)	γ	β, γ, δ'	
δ'	20.5	0.96 (*d*, *J* = 7.0)	γ	β, γ, δ	
Ala					
CO	174.4			PA_1-α, α	
α	49.1	5.75 (*q*, *J* = 6.5)	β	β, MeN	α, MeN
β	14.4	1.34 (*d*, *J* = 6.5)	α	α	
MeN	30.0	3.00 (*s*)		β	
PA_1					
CO	171.4			Trp-2, α, β	
α	49.6	5.50 (*d*, *J* = 6.5)	β	β, γ	β_2, MeN
β	23.4	1.97–2.00 (*m*, H_a)	α, β_2, γ		
		1.65–1.67 (*m*, H_b)	α, β_1, γ	α, γ, δ	
γ	21.0	1.63–1.65 (*m*, H_a)	β, γ_2, δ		
		1.46–1.49 (*m*, H_b)	β, γ_1, δ	α, β, δ	
δ	47.1	3.11–3.13 (*m*, H_a)	γ, δ_2		
		2.79–2.82 (*m*, H_b)	γ, δ_1	β, γ	
PA_2					
CO	171.1			Ala-α, MeN, α	
α	51.9	5.30 (br. *s*)	β	β, γ	β_2, Trp-8a
β	26.3	2.33–2.35 (*m*, H_a)	α, β_2, γ		
		1.59–1.61 (*m*, H_b)	α, β_1, γ	α, γ, δ	
γ	20.5	2.12–2.13 (*m*, H_a)	β, γ_1, δ		
		1.93–1.96 (*m*, H_b)	β, γ_2, δ	α, β, δ	
δ	46.7	3.11–3.13 (*m*, H_a)	γ, δ_2		
		2.80–2.82 (*m*, H_b)	γ, δ_1	β, γ	
PA_3					
CO	171.9			PA_2-α, α	
α	47.9	5.26 (br. *s*)	β	β, γ	β_2
β	24.4	2.33–2.35 (*m*, H_a)	α, β_2, γ		
		1.59–1.61 (*m*, H_b)	α, β_1, γ	α, γ, δ	
γ	19.8	2.12–2.13 (*m*, H_a)	β, γ_1, δ		
		1.93–1.96 (*m*, H_b)	β, γ_2, δ	α, β, δ	
δ	46.4	3.11–3.13 (*m*, H_a)	γ, δ_2		
		2.80–2.82 (*m*, H_b)	γ, δ_1	β, γ	
Trp					
CO	173.7			2, 3, Leu-α	
C(2)	61.5	4.81 (*d*, *J* = 8.0)	3		3-H_b
C(3)	40.0	2.48 (*d*, *J* = 14.0, H_a)	2, 3-H_a		2, 3-H_b
		2.35 (*dd*, *J* = 14.0, 8.0, H_b)	2, 3-H_b	2, 8a	2, 3-H_a
C(3a)	88.4			4, 2, 8a	
C(3b)	129.5			5, 7	

Table 1 (cont.)

Position	δ(C)	δ(H)	^{1}H,^{1}H-COSY	HMBC	NOESY
C(4)	123.7	7.22 (*d*, *J* = 8.0)	5	5	5
C(5)	119.2	6.81 (*dd*, *J* = 8.0,2.0)	4	7	4
C(6)	135.0			4, 7	
C(7)	110.7	6.73 (*s*)		5	
C(7a)	149.2			4	
C(8a)	85.6	5.49 (*s*)			3-H_a, MeN

[a]) Assignments are based on DEPT,^{1}H,^{1}H-COSY, HSQC, and HMBC experiments.

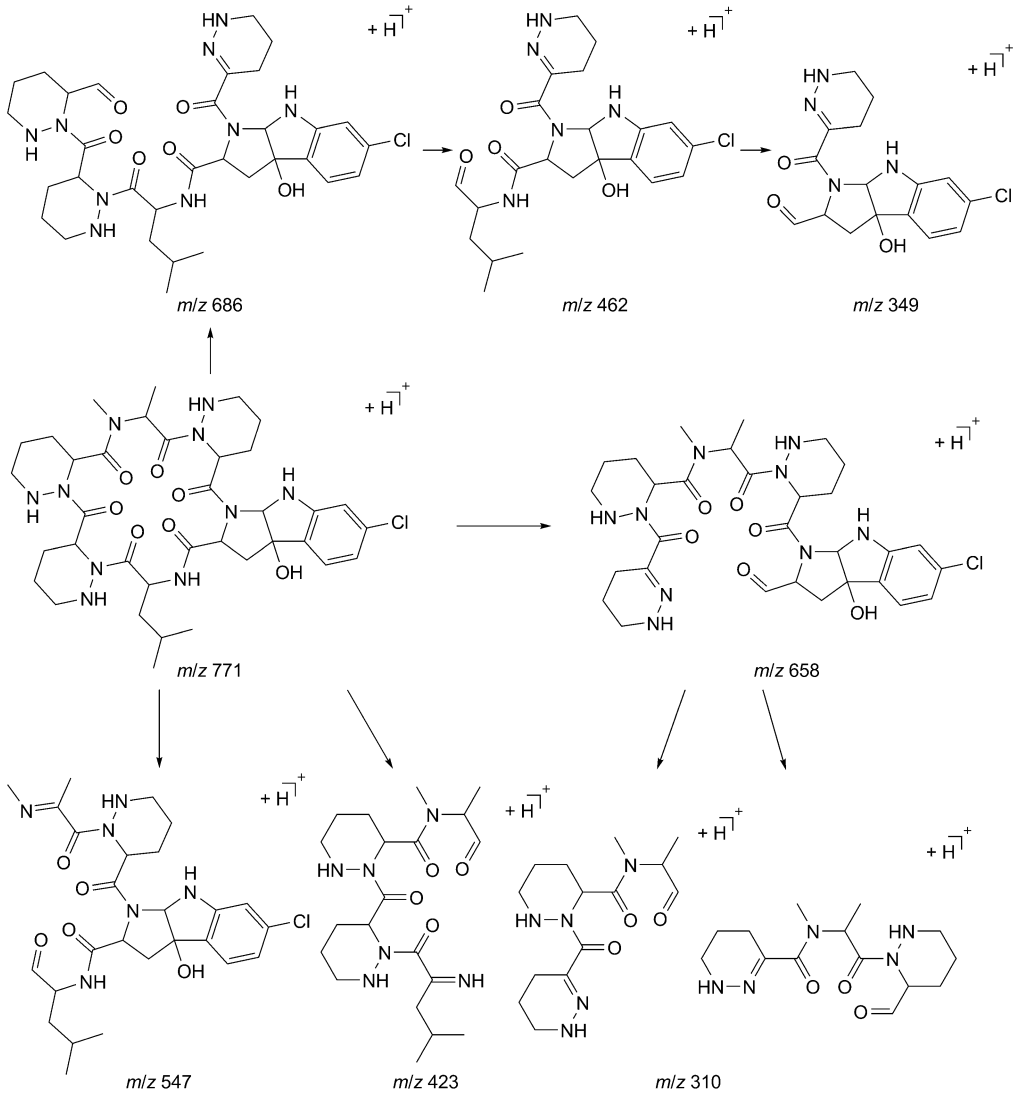
Table 2. *Characteristic MS/MS Fragments of NW-G01, and Compounds* **1** *and* **2**

Compound	$[M+H]^+$	Fragments of amino acids[a])						
		$P_1TLP_3P_2$ or $P_1T'VP_3P_2$	$P_3P_2AP_1T$ or $P_3P_2AP_1T'$	AP_1TL or $AP_1T'V$	LP_3P_2A or VP_3P_2A	P_2AP_1	P_1TL or $P_1T'V$	P_1T or P_1T'
NW-G01	757	672	658	533	409	310	448	349
1	771	686	658	547	423	310	462	349
2	773	688	674	549	409	310	464	365

[a])P_1, PA_1; P_2, PA_2; P_3, PA_3; T, Trp; V,Val; A, Ala; L, Leu; T′, Trp′.

exhibited signals of two sp^3 CH groups at δ(H) 5.27 (*d*, *J* = 8.0) and 2.02–2.04 (*m*), of two secondary Me groups at δ(H) 0.99 (*d*, *J* = 7.0) and 0.97 (*d*, *J* = 7.0), which were assigned to the α, β, λ, and λ' H-atoms of valine based on the HSQC and HMBC spectra of **2**, and by comparison with the corresponding chemical-shift and coupling-constant values of NW-G01. *N*-Me-alanine (MeAla) and three molecules of piperazic acid could also be identified on the basis of the HSQC and HMBC spectra of **2**, and by comparison with the corresponding chemical-shift and coupling-constant values of NW-G01 and **1**. The characteristic signals of a CH group at δ(H/C) 5.33/85.5, O-bearing quaternary C-atom at δ(C) 88.7, two aromatic CH groups at δ(H/C) 6.70/112.4, 6.89/111.0, and four sp^2 quaternary C-atoms at δ(C) 121.6, 130.8, 140.9, and 146.8 indicated the presence of a tryptophan derivative (Trp). Careful comparison of the DEPT spectra of **2** with those of NW-G01 indicated that there were two aromatic CH groups and four sp^2 quaternary C-atoms in **2**, whereas there were three aromatic CH groups and three sp^2 quaternary C-atoms in NW-G01, implying that one H-atom of aromatic CH group in **2** was replaced with a substituent. The molecular formula of **2** and NW-G01 were $C_{35}H_{49}ClN_{10}O_8$ and $C_{35}H_{49}ClN_{10}O_7$, respectively, indicating that **2** is the hydroxylated derivative of NW-G01. The cross peaks between H–C(4), and C(3a), C(3b), C(5), C(6), and C(7a), and between H–C(7), and C(3b), C(6), and C(7a) were observed in the spectrum of HMBC, revealing that the free OH group was at C(5) of Trp. Thus, the sixth amino acid was deduced as 6-chloro-1,2,3,3a,8,8a-hexahydro-3a,5-dihydroxy-pyrrolo[2,3-*b*]indole-2-carboxylic acid. The amino acids of **2** were established as *N*-methylalanine (MeAla), valine (Val), 6-chloro-1,2,3,3a,8,8a-hexahydro-3a,5-dihydroxy-pyrrolo[2,3-*b*]indole-2-carboxylic acid (Trp′), and three molecules of piperazic acid (PA).

Scheme 1. *Fragmentation Pathway of NW-G08* (**1**) *in Positive-Ion-Mode MS/MS*



The sequence linkage of the amino acids in **2** could be readily determined by comparison of the characteristic MS/MS fragmention peaks in the spectra of NW-G01 as described above (*Table 2*, *Scheme 2*, and *Fig. 2*). The common fragmention peaks of Val-PA_3-PA_2-Ala (*m/z* 409) and PA_2-MeAla-PA_1 (*m/z* 310) could be observed in the MS/MS spectra of **2** and NW-G01. Other fragmention peaks at *m/z* 688, 674, 549, 464, and 365 were observed in the spectrum of **2**; each fragment was 16 Da higher than the corresponding ions revealed in the spectrum of NW-G01, and they were assigned to PA_1-Trp′-Val-PA_3-PA_2, PA_3-PA_2-Ala-PA_1-Trp′, MeAla-PA_1-Trp′-Val, PA_1-Trp′-Val,

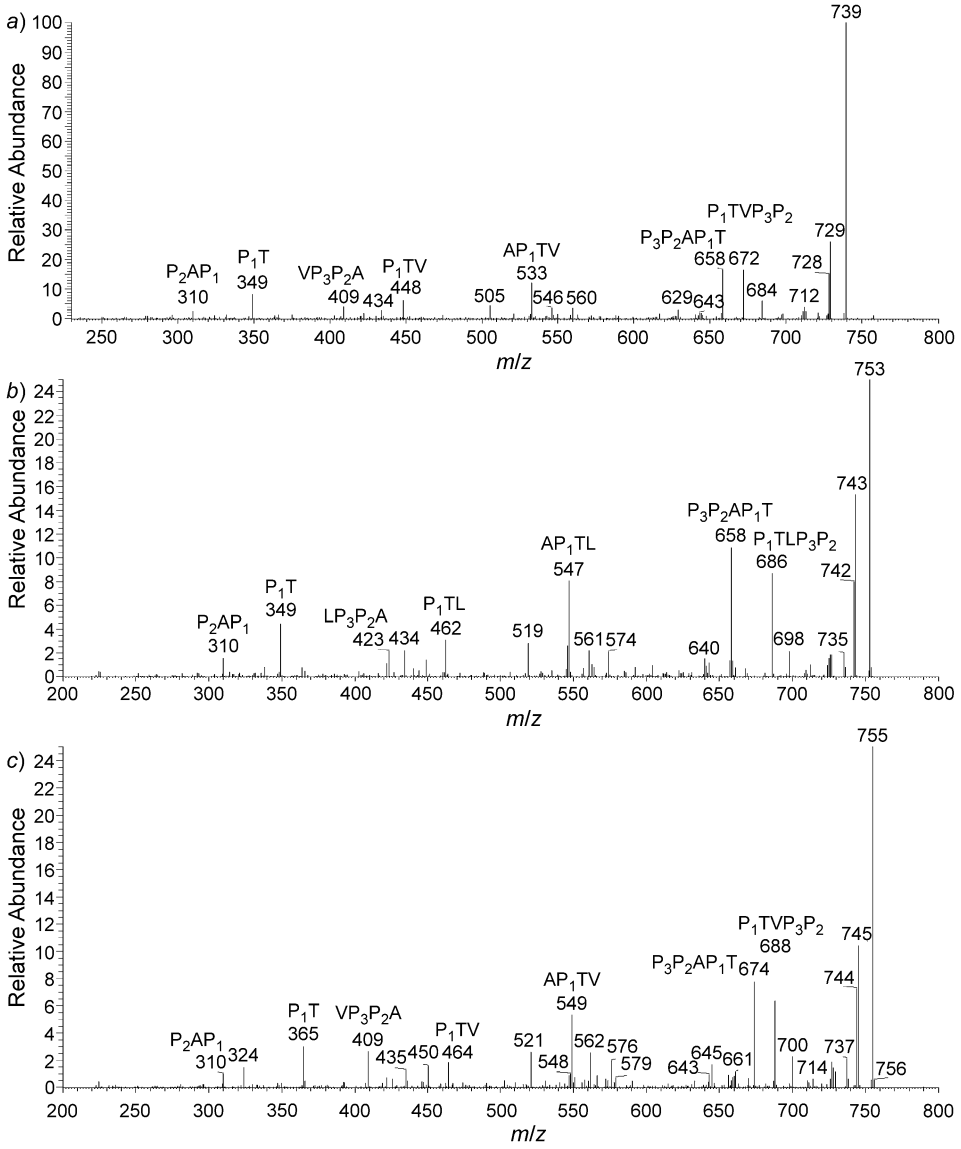


Fig. 2. *CID Spectra of a*) *NW-G01, b*) *NW-G08* (**1**)*, and c*) *NW-G09* (**2**) *in positive-ion-mode MS/MS*

PA_1-Trp′, respectively. Obviously, the sequence linkage of the amino acids in **2** was identical with that in NW-G01, except Trp in **2** was hydroxylated.

The absolute configurations of the amino acids were determined by the *Marfey*'s method as described above, as well as the NOESY spectrum. The amino acids in **2** were thus identified as D-valine, *N*-Me-D-alanine, (*3R*)-piperazic acid (PA_1), (*3R*)-piperazic

Table 3. *The ^{1}H- and ^{13}C-NMR Chemical Shifts of NW-G09*[a]) (**2**; CD_3OD, ^{1}H: 500 and ^{13}C: 125 MHz). δ in ppm, J in Hz.

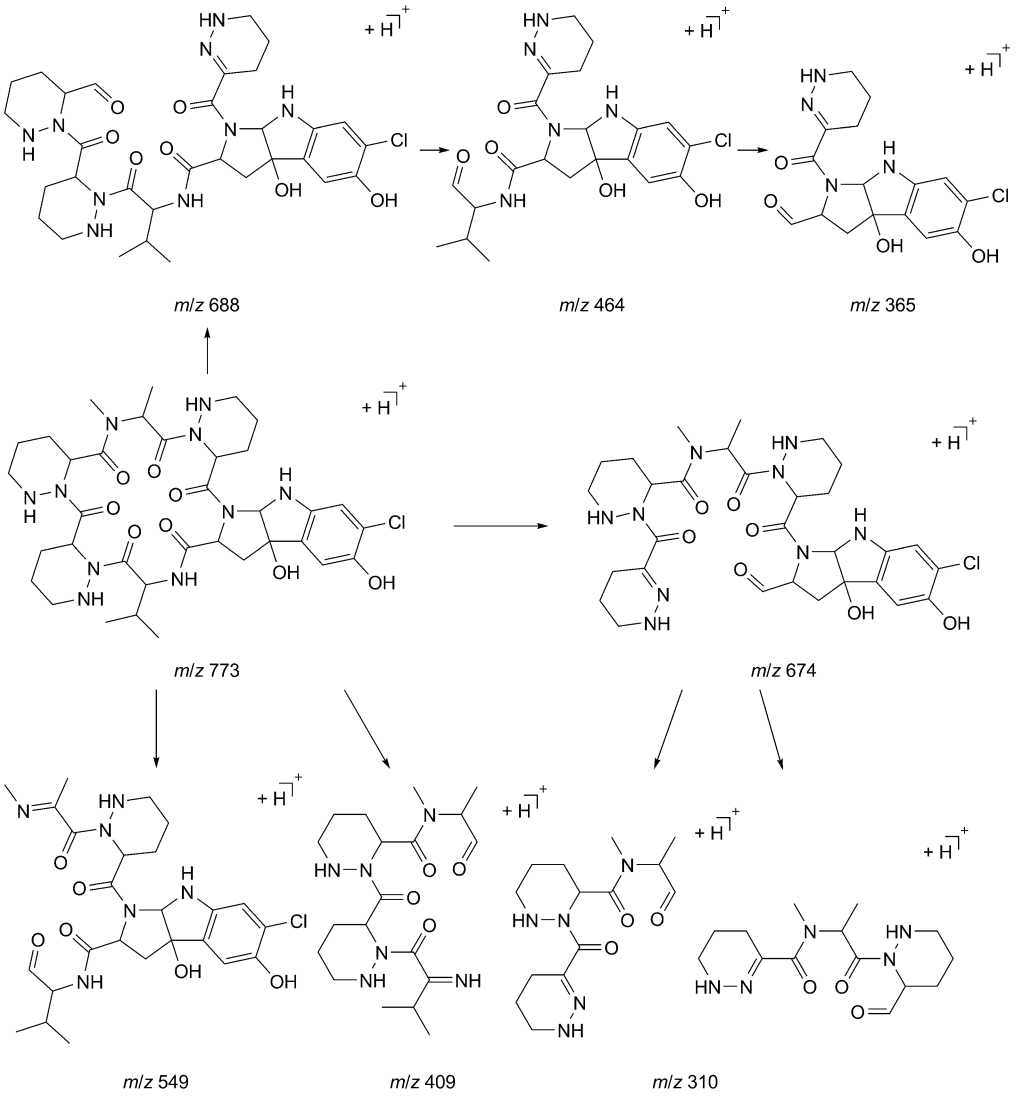
Position	δ(C)	δ(H)	^{1}H,^{1}H-COSY	HMBC	NOESY
Val					
CO	174.7			α, PA_3-α	
α	54.9	5.27 (d, J=8.0)	β, γ, γ'	β, γ, γ'	β, γ, γ'
β	29.9	2.02–2.04 (m)	α, γ, γ'	α, γ, γ'	
γ	17.4	0.99 (d, J=7.0)	α, β, γ'	α, β, γ'	
γ'	18.9	0.97 (d, J=7.0)	α, β, γ	α, β, γ	
Ala					
CO	174.2			PA_1-α, α	
α	48.5	5.75 (q, J=6.5)	β	β, MeN	α, MeN
β	13.8	1.34 (d, J=6.5)	α	α	
MeN	29.7	2.96 (s)		β	
PA_1					
CO	171.6			Trp-2,α, β	
α	49.9	5.51 (d, J=6.5)	β	β, γ	β_2, MeN
β	23.4	2.05–2.08 (m, H_a)	α, β_2, γ		
		1.76–1.80 (m, H_b)	α, β_1, γ	α, γ, δ	
γ	21.2	1.76–1.80 (m, H_a)	β, γ_2, δ		
		1.56–1.60 (m, H_b)	β, γ_1, δ	α, β, δ	
δ	47.3	3.14–3.17 (m, H_a)	γ, δ_2		
		2.80–2.82 (m, H_b)	γ, δ_1	β, γ	
PA_2					
CO	171.2			Ala-α,MeN, α	
α	52.3	5.38 (d, J=4.5)	β	β, γ	β_2, Trp-8a
β	25.1	2.30–2.33 (m, H_a)	α, β_2, γ		
		1.54–1.57 (m, H_b)	α, β_1, γ	α, γ, δ	
γ	19.9	2.13–2.15 (m, H_a)	β, γ_1, δ		
		1.95–2.00 (m, H_b)	β, γ_2, δ	α, β, δ	
δ	46.3	3.14–3.17 (m, H_a)	γ, δ_2		
		2.80–2.82 (m, H_b)	γ, δ_1	β, γ	
PA_3					
CO	172.3			PA_2-α, α	
α	47.3	5.22 (br. s)	β	β, γ	β_2
β	24.5	2.30–2.33 (m)	α, β_2, γ		
		1.54–1.57 (m)	α, β_1, γ	α, γ, δ	
γ	19.8	2.13–2.15 (m)	β, γ_1, δ		
		1.95–2.00 (m)	β, γ_2, δ	α, β, δ	
δ	46.4	3.14–3.17 (m)	γ, δ_2		
		2.80–2.82 (m)	γ, δ_1	β, γ	
Trp′					
CO	173.4			2, 3, Val-α	
C(2)	61.6	4.87 (d, J=8.0)	3	3	3-H_b
C(3)	39.6	2.54 (d, J=13.5, H_a)	2, 3-H_a		2, 3-H_b
		2.29 (dd, J=13.5, 8.0, H_b)	2, 3-H_b	2, 8a	2, 3-H_a
C(3a)	88.7			4, 2, 8a	
C(3b)	130.8			4, 7	
C(4)	111.0	6.89 (s)			
C(5)	140.9			4	

Table 3 (cont.)

Position	δ(C)	δ(H)	^{1}H,^{1}H-COSY	HMBC	NOESY
C(6)	121.6			4, 7	
C(7)	112.4	6.70 (*s*)			
C(7a)	146.8			4, 7	
C(8a)	85.5	5.33 (*s*)			3-H_a,MeN

[a]) Assignments are based on DEPT, ^{1}H,^{1}H-COSY, HSQC and HMBC experiments.

Scheme 2. *Fragmentation Pathway of NW-G09* (**2**) *in Positive-Ion-Mode MS/MS*



acid (PA_2), (*3S*)-piperazic acid (PA_3), and (2*S*,3a*R*,8a*S*)-6-chloro-1,2,3,3a,8,8a-hexahydro-3a,5-dihydroxy-pyrrolo-[2,3-*b*]indole-2-carboxylic acid (*Fig. 1*).

Antibacterial Activity. The antibacterial activities of **1** and **2** against several strains of bacteria were evaluated by micro-broth dilution method as described in [16]. The results indicated that **1** and **2** could effectively inhibit *Gram*-positive bacteria, such as *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRSA), whereas they were inactive to *Gram*-negative bacteria (*Table 4*). The minimum inhibition concentrations (*MIC*s) of **1** against *B. cereus*, *B. subtilis*, *S. aureus*, and MRSA were 6.25, 6.25, 1.56, and 1.56 μg/ml, respectively, of **2** 25.0, 25.0, 12.5, and 12.5 μg/ml, respectively, of NW-G01 12.5, 12.5, 6.25, and 6.25 μg/ml, respectively, and of ampicillin sodium were 6.25, 1.56, 6.25, and >100.0 μg/ml, respectively. Compound **1** showed stronger antibacterial activity than NW-G01; because the only difference between their structures was that valine in NW-G01 was replaced by leucine in **1**, it indicated that leucine was more beneficial to the activity than valine. The activity of **2** was slightly lower than that of NW-G01, revealing that the introduction of a free OH group at C(5) of Trp′ was not contributing to the activity. The structure–activity relationship of these three antibiotics could be explained by the hydrophobic character of the molecules. Compared with NW-G01, the incorporation of leucine into **1** increased the hydrophobic property, whereas the hydroxylation of Trp decreased the hydrophobic nature of **2**, evidencing that high hydrophobic nature was benefical for the activity. We assume that the higher hydrophobicity could promote the penetration of antibiotics through biomembrane, or increase the binding affinity with receptor. It is worth noting that these cyclic peptides showed the same sensitivity to *S. aureus* and MRSA. MRSA is of particular clinical importance, because it is not only predictably cross-resistant to all currently licensed β-lactam antibiotics, but it is also typically resistant to other multiple antibiotics. The results indicated that these cyclic peptides may have different mechanisms of action, so they could be potential candidates for the development of novel drugs.

Table 4. *MIC Values of Compounds* **1** *and* **2** *against Several Strains of Bacteria*

Tested bacteria	*MIC* [μg/ml]			
	1	**2**	NW-G01	Ampicillin
Bacillus cereus (CGMCC 1.1846)	6.25	25	12.5	6.25
Bacillus subtilis (CGMCC 1.88)	6.25	25	12.5	1.56
Staphylococcus aureus (CGMCC 1.89)	1.56	12.5	6.25	6.25
Escherichia coil (CGMCC 1.1574)	>100	>100	>100	3.13
Pseudomonas aeruginosa (CGMCC 1.2031)	>100	>100	>100	>100
MRSA	1.56	12.5	6.25	>100

Experimental Part

General. M.p.: *WPR* apparatus; uncorrected (*Shanghai Jingke Co.*).Optical rotations: *Horiba SEPA 300* polarimeter. UV Spectra: *Shimadzu UV-2401A* spectrometer. IR Spectra: *Nicolet FT-IR 750* spectrometer. ^{1}H- and^{13}C-NMR, DEPT, ^{1}H,^{1}H-COSY, HSQC, HMBC, and NOESY spectra: *Bruker Avance III-500* NMR spectrometer (*Bruker Daltonics Inc.*), δ in ppm, with TMS as internal standard,

and *J* in Hz (^{1}H: 500, ^{13}C: 125 MHz). ESI-MS/MS and HR-ESI-MS: *Finnigan LCQ Advantage* ion-trap mass spectrometer (*Thermo Fisher Co.*) and an *APEX II FT-ICR* mass spectrometer (*Bruker Daltonics Inc.*).

Microorganism and Fermentation. The producing strain *S. alboflavus* 313 was isolated from a soil sample collected in Qinling Mountain, Shaanxi Province, P. R. China, and identified by its morphology, physiology, biochemistry, and 16S rRNA gene sequence. The voucher specimen of this streptomycete was deposited with the Institute of Pesticide Science, Northwest A & F University, P. R. China.

The spores of *S. alboflavus* 313 grown on *Gause*'s No. 1 agar were used to inoculate in a 250-ml flask containing 50 ml of a sterile seed medium consisting of glucose (0.8%), soluble starch (0.8%), beef extract (0.6%), peptone (1.0%), and NaCl (0.5%); pH 7.2. The flask was shaken on a shaker at 180 rpm. for 24 h at 28°. Ten ml of the seed culture were transferred to 250-ml flasks containing 50 ml of a sterile producing medium consisting of glucose (3.0%), millet steep liquor (1.0%), peptone (1.5%), NaCl (0.5%), and $CaCO_3$ (0.5%); pH 7.2. Fermentation was carried out at 180 rpm. for 5 d at 28° on a rotary shaker.

Extraction and Isolation. The culture of 200 l was filtered through cheese cloth to separate the medium and culture liquid. The filtrate was absorbed onto *HPD400* macroporous resin (*Baoen Co., Ltd.*, Hebei, P. R. China), and then eluted with H_2O and MeOH in sequence. The MeOH fraction was evaporated in vacuum. The concentrate was subjected to column chromatography (CC; SiO_2 (600 g, 200–300 mesh, *Qingdao Marine Chemical Co., Ltd.*, Shandong, P. R. China); AcOEt/MeOH 75:25 (*v*/*v*)). The antimicrobial fraction was concentrated in vacuum and then subjected to flash chromatography (FC; *ODS-AP* (50 μm, *Daiso Co., Ltd.*, Osaka, Japan); eluted with the mixture of MeOH/H_2O at the ratio of 60:40 (*v*/*v*)). The active fractions were concentrated in vacuum and further purified on a *Shimadzu 6AD* HPLC apparatus (*Shimadzu Co., Ltd.*, Tokyo, Japan) equipped with a column of *Hypersil ODS-BP* (20 × 250 mm, 10 μm, flow rate 8.0 ml/min) to afford two novel cyclic hexapeptides, NW-G08 (**1**; 8.0 mg) and NW-G09 (**2**; 7.6 mg).

Marfey*'s Analysis:* Approximately 1.0 mg of tested sample was hydrolyzed with 100 μl of 6N HCl at 110° for 24 h. The acid hydrolysate was evaporated to dryness and dissolved in 100 μl of 0.1N HCl. To 50 μl of the acidic soln., 80 μl of 1N $NaHCO_3$ and 400 μg of 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA; *Marfey*'s reagent) with 40 μl of acetone was added, the mixture was then heated at 50° for 1 h. After cooling the mixture to r.t., it was neutralized with 40 μl of 1N HCl and evaporated to dryness. The residue was dissolved in 40 μl of MeCN, and the FDAA derivative soln. was analyzed by RP-HPLC. The analysis of the derivatives was performed on a *Hypersil ODS-BP* (250 × 4.6 mm, 5 μm) column maintained at 30° with UV detector at 340 nm. The mixture MeCN/50 mMEt_3N phosphate buffer (pH 5.0) 40:60 (*v*/*v*) was used as mobile phase, and the flow rate was 1.0 ml per min. The FDAA derivatives of the acid hydrolysate were identified by comparing the retention times with FDAA-derivatized standard amino acids.

Antibacterial Assay. The standard bacterial strains *Bacillus cereus* (1.1846), *Bacillus subtilis* (1.88), *Staphylococcus aureus* (1.89), *Escherichia coil* (1.1574), and *Pseudomonas aeruginosa* (1.2031) were obtained from *China General Microbiological Culture Collection Center*. A clinical isolate of MRSA was obtained from Nanjing Medical University. Ampicillin sodium (*Sigma*, Shanghai, P. R. China) was used as positive control. The antibacterial activities of compounds against six strains of bacteria were evaluated by the micro-broth dilution method in 96-well plates [16]. The inoculum was prepared by suspending several colonies from an overnight culture of tested bacteria from 0.5% sheep blood agar media in *Müller–Hinton* broth (*Hangzhou Microbial Reagent Co. Ltd.*, Zhejiang, P. R. China) and adjusting to a 0.5 *McFarland* standard (*ca.* 1.5×10^8 colony-forming units per ml). A further dilution of 1:200 was performed by placing 0.25 ml of the adjusted suspension into 49.75 ml of *Müller–Hinton* broth. The tested compounds were first dissolved in DMSO at the concentration of 10 mg/ml, and it was diluted ten-fold with sterile H_2O to give the stock soln. Twofold serial dilutions of the tested compounds were prepared in *Müller–Hinton* broth. Then, the dilutions and inoculated suspension of the bacteria were delivered to wells of a 96-well plate at the ratio of 1:1. The final concentration of inoculum in each well was 3.7×10^5 colony-forming units per ml. After incubation for 24 h at 30°, the *MIC*s were examined. Experiments were run in triplicate, and standard ampicillin sodium was used as the positive control.

NW-G08 (=*(4aS,10aR,13S,19aR,21aS,26bR,27aS,30S)-24-Chloro-2,3,4,4a,8,9,10,10a,12,13,17,18,19,19a,22,26b,27,27a,29,30-icosahydro-26b-hydroxy-12,13-dimethyl-30-(2-methylpropyl)-7*H,*21a*H-*tripyridazino[1'',6'':10',11';1''',6''':16',17';1'''',6'''':7',8'][1,4,7,10,13,16]hexaazacyclooctadecino[1',2':1,5]pyrrolo[2,3-*b*]indole-5,11,14,20,28,31(1*H,*16*H)-*hexone*; **1**). Amorphous white powder. M.p. 192.2–194.0°. $[\alpha]_D = -44.5$ ($c = 0.25$, MeOH). UV (MeOH): 205, 235. IR (KBr): 3397, 2956, 2933, 1660, 1415, 1336, 1249, 1072, 752.^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS (pos.): 771.3716 ($[M+H]^+$, $C_{36}H_{52}ClN_{10}O_7^+$; calc. 771.3709).

NW-G09 (=*(4aS,10aR,13S,19aR,21aS,26bR,27aS,30R)-24-Chloro-2,3,4,4a,8,9,10,10a,12,13,17,18,19,19a,22,26b,27,27a,29,30-icosahydro-25,26b-dihydroxy-12,13-dimethyl-30-(propan-2-yl)-7*H,*21a*H-*tripyridazino[1'',6'':10',11';1''',6''':16',17';1'''',6'''':7',8'][1,4,7,10,13,16]hexaazacyclooctadecino[1',2':1,5]pyrrolo[2,3-*b*]indole-5,11,14,20,28,31(1*H,*16*H)-*hexone*; **2**). Amorphous white powder. M.p. 225.0–226.0°. $[\alpha]_D = -28.0$ ($c = 0.25$, MeOH). UV (MeOH): 205, 240. IR (KBr): 3427, 2960, 2931, 1636, 1443, 1407, 1335, 1248, 1155, 1084, 642.^{1}H- and ^{13}C-NMR: see *Table 3*. HR-ESI-MS (pos.): 773.3511 ($[M+H]^+$, $C_{35}H_{50}ClN_{10}O_8^+$; calc. 773.3502).

This study was supported part by the grant of the *National Key Basic Research Program* (973 Program, 2010CB126100) from *Science and Technology Ministry of China*, the *National Natural Science Foundation of China* (No.30971935), *Postdoctoral Science Foundation of China* (20100471644 and 201104683), and *Program for New Century Excellent Talents in University from Education Ministry of China*.

REFERENCES

[1] J. Bérdy, *J. Antibiot.* **2005**, *58*, 1.
[2] C. H. Hassall, K. E. Magnus, *Nature* **1959**, *184*, 1223.
[3] T. A. Smitka, J. B. Deeter, A. H. Hunt, F. P. Mertz, R. M. Ellis, L. D. Boeck, R. C. Yao, *J. Antibiot.* **1988**, *41*, 726.
[4] H. Maehr, C.-M. Liu, N. J. Palleroni, J. Smallheer, L. Todaro,T. H. Williams, J. F. Blount, *J. Antibiot.* **1986**, *39*, 17.
[5] Y. Sakai, T. Yoshida, T. Tsujita, K. Ochiai, T. Agatsuma, Y. Saitoh, F. Tanaka, T. Akiyama, S. Akinaga, T. Mizukami, *J. Antibiot.* **1997**, *50*, 659.
[6] R. P. Maskey, S. Fotso, M. Sevvana, I. Usón, I. Grün-Wollny, H. Laatsch, *J. Antibiot.* **2006**, *59*, 309.
[7] K. Umezawa, Y. Ikeda, Y. Uchihata, H. Naganawa, S. Kondo, *J. Org. Chem.* **2000**, *65*, 459.
[8] K. S. Lam, G. A. Hesler, J. M. Mattei, S. W. Mamber, S. Forenza, K. Tomita, *J. Antibiot.* **1990**, *43*, 956.
[9] J. E. Leet, D. R. Schroeder, B. S. Krishnan, J. A. Matson, *J. Antibiot.* **1990**, *43*, 961.
[10] J. E. Leet, D. R. Schroeder, J. Golik, J. A. Matson, T. W. Doyle, K. S. Lam, S. E. Hill, M. S. Lee, J. L. Whitney, B. S. Krishnan, *J. Antibiot.* **1996**, *49*, 299.
[11] E. D. Miller, C. A. Kauffman, P. R. Jensen, W. Fenical, *J. Org.Chem.* **2007**, *72*, 323.
[12] A. J. Oelke, D. J. France, T. Hofmann, G. Wuitschik, S. V. Ley, *Nat. Prod. Rep.* **2011**, *28*, 1445.
[13] Z. Guo, L. Shen, Z. Ji, J.Zhang, L. Huang, W. Wu, *J. Antibiot.* **2009**, *62*, 201.
[14] Z. Guo, Z. Ji, J. Zhang, J. Deng, L. Shen, W. Liu, W. Wu, *J. Antibiot.* **2010**, *63*, 231.
[15] Z. Guo, Z. Ji, J. Zhang, J. Deng, L. Shen, W. Liu, W. Wu, *J. Antibiot.* **2010**, *63*, 733.
[16] Z. Ji, M. Wang, J. Zhang, S. Wei, W. Wu, *J. Antibiot.* **2007**, *60*, 739.

Received November 5, 2011